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2 **Marek's disease virus-encoded miR-155 ortholog critical for the**
3 **induction of lymphomas is not essential for the proliferation of**
4 **transformed cell lines**

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23

24 **Running title:** MDV-miR-M4 not needed for maintaining transformation

25

26 **Abstract**

27 MicroRNAs (miRNAs) are small non-coding RNAs with profound regulatory roles in
28 many areas of biology, including cancer. MicroRNA 155 (miR-155), one of the
29 extensively studied multifunctional miRNAs, is important in several human malignancies
30 such as diffuse large B cell lymphoma and chronic lymphocytic leukemia. Moreover,
31 miR-155 orthologs KSHV-miR-K12-11 and MDV-miR-M4, encoded by Kaposi's
32 sarcoma-associated herpesvirus (KSHV) and Marek's disease virus (MDV) respectively,
33 are also involved in oncogenesis. In MDV-induced T-cell lymphomas and
34 lymphoblastoid cell lines derived from them, MDV-miR-M4 is highly expressed. Using
35 excellent disease models of infection in natural avian hosts, we showed previously that
36 MDV-miR-M4 is critical for the induction of T-cell lymphomas as mutant viruses with
37 precise deletions were significantly compromised in their oncogenicity. However, these
38 studies did not elucidate whether continued expression of MDV-miR-M4 is essential for
39 maintaining the transformed phenotype of tumor cells. Here using an *in situ*
40 CRISPR/Cas9 editing approach, we deleted MDV-miR-M4 from the MDV-induced
41 lymphoma-derived lymphoblastoid cell line MDCC-HP8. Precise deletion of MDV-miR-
42 M4 was confirmed by PCR, sequencing, quantitative RT-PCR and functional analysis.
43 Continued proliferation of the MDV-miR-M4-deleted cell lines demonstrated that MDV-
44 miR-M4 expression is non-essential for maintaining the transformed phenotype, despite
45 its initial critical role in the induction of lymphomas. Ability to examine the direct role of
46 oncogenic miRNAs *in situ* in tumour cell lines is valuable in delineating distinct
47 determinants and pathways associated with the induction or maintenance of

48 transformation in cancer cells and will also contribute significantly to gain further
49 insights into the biology of oncogenic herpesviruses.
50

51 **Importance**

52 Marek's disease virus (MDV) is an alphaherpesvirus associated with Marek's disease, a
53 highly contagious neoplastic disease of chickens. MD serves as an excellent model for
54 studying virus-induced T-cell lymphomas in the natural chicken hosts. Among the limited
55 set of genes associated with MD oncogenicity, MDV-miR-M4, a highly expressed viral
56 ortholog of the oncogenic miR-155, has received extensive attention due to its direct role
57 in the induction of lymphomas. Using a targeted CRISPR-Cas9-based gene editing
58 approach in MDV-transformed lymphoblastoid cell lines, we show that MDV-miR-M4,
59 despite its critical role in the induction of tumours, is not essential for maintaining the
60 transformed phenotype and continuous proliferation. As far as we know, this is the first
61 study where precise editing of an oncogenic miRNA has been carried out *in situ* in MD
62 lymphoma-derived cell lines to demonstrate that it is not essential in maintaining the
63 transformed phenotype.

64

65 Introduction

66 MicroRNAs (miRNAs) are ~22-nucleotide small RNA molecules that function as master
67 regulators of gene expression in many species including plants, worms, flies, animals, as
68 well as in a number of viruses. Most of the virus-encoded miRNAs are seen in DNA
69 viruses, with members of the family Herpesviridae accounting for the vast majority
70 demonstrating the significance of miRNA-mediated gene regulation in the biology of
71 herpesvirus infection (1-3). Identification of miRNAs encoded by human oncogenic γ -
72 herpesviruses such as Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr
73 virus (EBV) as well as avian oncogenic α -herpesvirus Marek's disease virus (MDV) has
74 highlighted the potential contribution of the virus-encoded miRNAs towards the
75 oncogenicity of these viruses. Among the several roles of the herpesvirus-encoded
76 miRNAs such as immune evasion, control of viral latency/lytic replication and oncogenic
77 potential (4-6), the role of viral orthologs of host miR-155 encoded by KSHV and MDV
78 in oncogenesis has been most extensively studied (5, 7). As a multifunctional miRNA
79 expressed primarily in the hematopoietic and cells of the immune systems, miR-155 is
80 highly conserved in most species including humans and chickens, and are associated with
81 different lymphomas (8-11). In EBV-induced B-cell transformation as well as in a
82 number of EBV-associated B-cell lymphomas including Hodgkin's lymphoma, diffuse
83 large B-cell lymphoma (DLBCL) and Burkitt's lymphoma in humans, upregulation of
84 miR-155 resulting in escalated cell proliferation and neoplastic transformation has been
85 reported (12, 13). KSHV, a human gammaherpesvirus associated with
86 lymphoproliferative disorders such as primary effusion lymphoma (PEL), multicentric
87 Castleman disease (MCD) and B lymphomagenesis in AIDS patients, encodes 25

88 miRNAs. Among these miRNAs, KSHV-K12-11 that plays critical role in pathogenesis
89 is a functional ortholog of hsa-miR-155 sharing identical seed sequences (14-16). MDV
90 encodes MDV-miR-M4-5p (miR-M4), a functional ortholog with identical seed
91 sequences with miR-155 and KSHV-K12-11 that has been shown to play a critical role in
92 the induction of lymphomas (6).

93 Marek's disease (MD) is a lymphoproliferative disease of chickens characterized by
94 rapid-onset lymphomas in multiple organs, and infiltration into peripheral nerves causing
95 paralysis. MD serves as an excellent model for studying virus-induced T-cell lymphomas.
96 Among the more than 100 genes encoded by the MDV (17, 18), the basic leucine zipper
97 protein Meq (MDV EcoRI Q), which is expressed both in lytic and latent infections
98 undisputedly, is the most important viral gene associated with MD oncogenicity (19, 20).
99 Deleting the Meq gene or inhibition of its important interactions with host proteins such
100 as c-Jun, c-Fos and C-terminal binding protein (CtBP) can affect the oncogenicity of the
101 virus (21-23). Although the viral telomerase RNA (vTR) also has been shown to promote
102 MDV-induced oncogenesis (24), the role of MDV-encoded miRNAs in oncogenesis has
103 drawn extensive attention (25-27). MDV encodes 14 miRNA precursors producing 26
104 mature miRNAs which are clustered into three separate genomic loci within the repeat
105 regions of the viral genome. MDV-miR-M4, located in the cluster 1, was shown to be the
106 viral ortholog of miR-155 (28). The oncogenic property of miR-155 together with the
107 observation of high level of miR-M4 expression in tumor cells and the identification of
108 several cancer pathway-related target genes suggested the important role of this miRNA
109 in MDV-induced oncogenesis. Indeed, we and others have previously demonstrated the
110 direct role of miR-M4 in the induction of tumours using recombinant MDV engineered to

111 have deletion- or seed region- mutations in miR-M4 using *in vivo* experiments in
112 chickens (6, 29). Furthermore, we showed that the loss of oncogenic phenotype of miR-
113 M4-deletion mutant of MDV can be partially rescued by MDV expressing gga-miR-155
114 demonstrating the similarities in the function of the two orthologs (6). While the role of
115 miR-M4 in the induction of MD lymphomas has been clearly demonstrated in these
116 studies, it remains unclear whether continued high level expression of miR-M4 is
117 essential for maintaining the transformed phenotype of MDV-transformed tumour cells.
118 As clonal populations of transformed tumor cells with latent MDV genome and limited
119 gene expression (30-32), lymphoblastoid cell lines (LCL) derived from MD lymphomas
120 have served as valuable resources to understand distinct aspects of virus-host interactions
121 in transformed cells. However detailed investigations into the role of different viral and
122 host determinants in these cells have been difficult due to the lack of tools for
123 manipulation of viral/host genomes of these cells *in situ*. Following our recent success in
124 efficient editing of the MDV genome in cell culture systems *in vitro* that supports lytic
125 virus replication (33), we explored the use of a gene editing approach in MDCC-HP8 cell
126 line that is latently infected with GA strain of MDV. Using MDCC-HP8 cells that stably
127 expressed Cas9 and synthetic gRNAs with two-part guide RNA system, we examined the
128 effect of deleting miR-M4 to gain insights into its functional role. Continued proliferation
129 of the miR-M4 knock-out cell lines suggested that expression of miR-M4 gene is not
130 essential for maintenance of the transformed state of the tumor cell line MDCC-HP8,
131 despite its known critical role in the induction of MD lymphomas

132 **Results**

133 **Knockout of MDV-miR-M4 in HP8 cells**

134 Based on the success of efficient editing of the MDV genome during lytic replication in
135 infected chicken embryo fibroblast (CEF) cultures in our previous studies (33), we
136 attempted editing of the latent MDV genome in virus-transformed cell lines. Initial
137 attempt with transfected gRNA-Cas9-expression plasmid showed low editing efficiency,
138 thought to be largely due to the relatively low transfection efficiency of the hard-to-
139 transfect MDV-transformed cell lines (data not shown). New gene editing strategy
140 involving the transfection of synthetic gRNAs with two-part guide RNA system into
141 MDV-transformed cell line stably expressing Cas9 (HP8-Cas9) showed great success.
142 For the targeted editing of the MDV-miR-M4 in the latent viral genome in this cell line,
143 two gRNAs M4-gN and M4-gC were designed using CRISPR guide RNA designing
144 software (<http://crispr.mit.edu/>). M4-gN targeted the upstream sequence of the mature
145 miR-M4 sequence and M4-gC targeted the sequence spanning the mature miR-M4
146 sequence and the loop region of the pre-miRNA hairpin structure resulting in the
147 predicated cleavage site exactly lies at the end of the miR-M4 mature sequence (Fig. 1a
148 & 1b). Successful miR-M4 deletion would release a 54-nt fragment following the
149 successful cleavage of the sequence by the two gRNAs. Considering the presence of
150 several MDV genomes integrated in multiple chromosomes of the chicken genome based
151 on fluorescence *in situ* hybridization (FISH) analysis (unpublished data) and the location
152 of miR-M4 in the terminal repeat region which doubles the copy number of miR-M4, two
153 distinct bands are expected with PCR tests on the genomic DNA from cells harvested 48
154 h after transfection using specific primers located at the flanking region of Cas9 targeting
155 sites. The top band of around 205-bp represented the unedited sequence or edited target
156 site/s with small indels if the two sites are not cleaved simultaneously. The bottom

157 smaller band of around 151-bp product corresponded to the edited region with 54bp
158 deletion between the two Cas9 cleavage sites. Interestingly, only the bottom band was
159 detected by PCR analysis indicating the highly efficient cleavage with the two gRNAs
160 with the majority of the cells transfected and edited efficiently. Despite of observation of
161 single band, single cell sorting was carried out to obtain pure population of miR-M4
162 deleted cell. Although only bottom band was obtained by PCR before sorting in the
163 mixed population, clones with top band were predominant after single cell cloning (Fig.
164 1c). Sequence analysis of four bottom bands confirmed that it represented the direct end
165 joining product of two predicted Cas9 target sites (Fig. 1a). Interestingly, the sequences
166 of all these four clones were identical suggesting that further screening of several
167 additional clones may be required to identify variations within the edited sequences. The
168 successful knockout of miR-M4 sequence was further confirmed by qRT-PCR analysis,
169 using uninfected CEF as a negative control. As expected, miR-M4 was absent from all
170 four miR-M4-deleted HP8 clones and control CEF, compared to the high level expression
171 detected in the parental HP8-Cas9 cells (Fig. 1d). These experiments demonstrated that
172 miR-M4 has been deleted successfully with two-part guide RNA system in HP8 cell line
173 stably expressing Cas9.

174 **miR-M4 is not essential in maintaining the transformed phenotype of MDV-** 175 **transformed cell line**

176 miR-M4 has been shown to be essential for the MDV in inducing tumors (6, 29). To
177 explore the role of miR-M4 in maintaining the transformed state, we examined the effect
178 of deletion of miR-M4 on the proliferation of HP8 cells. For this, we carried out kinetic
179 monitoring of proliferation of the wild type HP8-Cas9 and the miR-M4 deleted clones

180 using IncuCyte S3 Live-Cell Imaging system. The cell proliferation data in real time from
181 the images collected at 4 hours intervals showed that the miR-M4-deleted clones
182 proliferated at a significantly higher rate within the first three days compared to parental
183 HP8-Cas9 cells although different clones showed different levels of significance at
184 various time points. These results suggested that expression of miR-M4 was not essential
185 for the proliferation phenotype of these transformed cells.

186 **Pu.1 is up-regulated in HP8-ΔmiR-M4 cells**

187 Having shown that miR-M4 can be deleted from HP8 cell line and that it is not essential
188 for the continued proliferation of these transformed cells, we wanted to examine the
189 effect of miR-M4 deletion on the expression of its target proteins. For this, we chose to
190 analyze the expression levels of Pu.1, one of the very well characterised and validated
191 miR-M4 target (28). This was first assessed using luciferase reporter assay by
192 transfection of the reporter construct containing the wild-type predicted miR-M4-
193 response element (MRE) or the mutant MRE region of the 3' UTR of Pu.1 into the miR-
194 M4-deleted and the parental HP8-Cas9 cells. This assay showed that the relative Renilla
195 luciferase levels of reporter constructs with wild-type MRE sequences were reduced by
196 nearly 40% compared with the mutant MRE construct in the parental HP8-Cas9 cells.
197 Compared to this, such reduction of luciferase levels was absent in all of the miR-M4
198 deleted clones (Fig. 3a) demonstrating the functional effect of miR-M4 deletion on the
199 Pu.1 target. Next, we determined the miR-M4-mediated silencing by directly measuring
200 the level of Pu.1 expression in one of the selected mutant clones C48, along with the
201 parental cells. Immunoprecipitation-Western blot analysis showed that Pu.1 expression
202 level was much higher in miR-M4-deleted cells compared with the parental cells (Fig.

203 3b). Results from the reporter assay and the direct expression analysis of the Pu.1 target
204 have thus confirmed the deletion and functional consequences of miR-M4 in the mutant
205 C48 clone.

206 **Effect of miR-M4 deletion on expression of other viral miRNAs and Meq protein**

207 Having demonstrated successful knockout of miR-M4 from MDV genome in HP8 cell
208 line, we next analyzed the effect of miR-M4 deletion on expression of other MDV-
209 encoded miRNAs and the major viral oncoprotein Meq. The 14 MDV-encoded miRNA
210 precursors are clustered into three separate genomic loci. Cluster 1 (Meq cluster)
211 containing miR-M2, 3, 4, 5, 9 and 12 located upstream of Meq gene. The mid-cluster
212 containing three miRNA precursors (miR-M11, 31 and 1) located downstream of Meq.
213 The third cluster, referred to LAT-cluster, lies within the first intron of latency-associated
214 transcript (LAT). To assess the potential effect of miR-M4 deletion on other miRNAs, we
215 first amplified the cluster 1 miRNAs by PCR with the primers at the flanking region of
216 the cluster. Sequence of the PCR product was determined to confirm the absence of any
217 changes (data not shown) except for the edited region as shown in Figure 1a. Next we
218 analysed the expression of each miRNA in cluster 1, miR-M31 from cluster 2, miR-M6
219 and miR-M8 from cluster 3 using the RNA extracted from miR-M4-deleted clone 48 and
220 the parental HP8-Cas9. The host miRNA gga-let-7a was also measured, with total RNA
221 from uninfected CEF used as control. As shown in Figure 4a, all viral miRNAs were
222 absent and only let-7a was detectable in the CEF sample. Except for the absence of miR-
223 M4 from miR-M4-deleted clone 48, both the viral and the host miRNAs were detected in
224 HP8 before or after miR-M4 deletion. Quantitation of selected viral miRNAs by qRT-
225 PCR indicated that they are still expressed in the miR-M4-deleted clone 48, although

226 their expression levels showed variation compared to the parental HP8 cells (Fig. 4a). We
227 also examined Meq expression in the miR-M4-deleted cells by western blot analysis. An
228 ALV-transformed B-cell line HP45 and uninfected CEF which do not express Meq were
229 used as negative controls. Results of the western blot analysis confirmed the expression
230 of Meq in the miR-M4-deleted cells, demonstrating that miR-M4 was not required for
231 Meq expression in these cells (Fig. 4b).

232 ***v-rel* relieves the inhibition of miR-155 expression in HP8-ΔmiR-M4**

233 We have previously shown that miR-155 is consistently downregulated in MDV-
234 transformed tumours and cell lines (34) and this downregulation can be rescued by
235 expressing *v-rel* that also activate the expression levels of miR-M4 in these cells (35).
236 We wanted to examine whether the downregulation of miR-155 can be rescued without
237 the activation of miR-M4 by transduction of *v-rel* with RCAS(A)-*v-rel*-GFP virus in
238 HP8-ΔmiR-M4 clone 48. The GFP marker allowed sorting of the RCAS-infected cells.
239 Analysis of the sorted cells by Western blotting confirmed the expression of *v-rel*-GFP in
240 RCAS(A)-*v-rel*-GFP-infected cells and GFP expression in RCAS(A)-GFP infected cells
241 (Fig. 5a). Expression of *v-rel* increased the level of miR-155 expression by
242 approximately 6026-fold in HP8-ΔmiR-M4 cells but only 25-fold in HP8-Cas8 cells (Fig.
243 5b), demonstrating that deletion of miR-M4 increased miR-155 expression induced by *v-*
244 *rel*.

245 **Discussion**

246 Virus-host interactions in herpesviruses are characterized by long term survival as latent
247 infections in different cell types. With total dependence on the host cell, several viruses
248 have adopted strategies to modulate the host cellular environment, including the

249 modulation of miRNAs. A number of studies have demonstrated the role of miRNAs in
250 replication, pathogenesis and oncogenesis of herpesviruses (3, 4, 7, 36-38). These
251 included our own studies demonstrating the critical role of miR-M4 in the induction of
252 lymphomas by MDV (6). While these observations have also been confirmed by other
253 studies (29), the role of viral miRNAs in maintaining the transformed state, as well as in
254 other functions such as the switch of latency/lytic replication in tumor cells have not been
255 examined. Particularly, the role of miR-M4, the viral ortholog of oncogenic miR-155
256 encoded by oncogenic MDV, in maintaining the transformed phenotype of the tumor cell
257 line is unknown. MDV-transformed LCLs derived from MD lymphomas which contain
258 multiple copies of MDV genome integrated in different chromosomes are valuable to
259 study latency, transformation and reactivation *in situ*. Having established the
260 CRISPR/Cas9-based editing of the viral genome at relatively high efficiency in MDV-
261 transformed cell lines, we report here the precise knockout of miR-M4 from the MDV
262 genome in the LCL HP8. Results from these studies show that miR-M4, despite its
263 critical role in the induction of lymphomas by oncogenic MDV strains, is not required for
264 the continued proliferation of MDV-transformed HP8 LCL. As far as we know, this is the
265 first study that makes use of the CRISPR/Cas9-based gene editing technology *in situ* to
266 demonstrate that a critical virus-encoded miRNA is not essential to maintain the
267 transformed phenotype of a virus-induced cancer cell line.

268 By transfection of two parts synthetic gRNA into HP8 cells stably expressing Cas9, we
269 have shown here that miR-M4 can be deleted at a relatively high efficiency (Fig. 1c).
270 Considering the presence of the multiple copies of the target loci in these cell lines, the
271 high editing efficiency highlighted that efficient gRNA, rather than the copy numbers of

the target genes, is the key to achieve the desired editing even in the hard-to-transfect cell lines such as the MDV-transformed LCL. Although the editing efficiency based on the PCR test on the transfected cell lines appeared to be very high, sorting of the single cell populations did identify a number of unedited clones, further highlighting the importance of single cell sorting in gene editing pipelines. These findings are also consistent with our observation that the recovery rate of edited cells is probably much lower than that of the unedited cell populations, suggesting that single cell cloning is a required step to get the pure populations of the edited cells regardless of the efficiency of gene editing. The successful knockout of miR-M4 demonstrated the value of this approach in identifying other molecular determinants associated with different phenotypes including latency/lytic switch in LCLs. While the growth of the miR-M4-deleted cells confirmed that the expression of miR-M4 is not essential to maintain the transformation and proliferation of LCL, Δ miR-M4 cell line that we have generated will also be a valuable research tool for addressing significant biological questions in the future on the functional role of this important miRNA homolog. For example, it will be interesting to know if the populations of shared target genes of MDV-miR-M4, miR-155 and KSHV-miR-K12-11 (5) are upregulated in the miR-M4-deleted cells and downregulated after *v-rel* transduction which activates miR-155 expression (Fig. 5b). Similarly, future studies on the global analysis of the changes in the transcriptome and proteomes of the edited cell populations, together with changes in the viral and host epigenomes will throw more insights into the fine tuning of the molecular regulatory network around these family of miRNAs in these virus-transformed cell lines. Finally, these cells also give the opportunity to investigate

294 the role of miR-M4 to induce lymphomas (transplantable tumors) *in vivo* in
295 experimentally-infected target chicken hosts.

296 Repair by non-homologous end joining (NHEJ) is usually accompanied by random
297 nucleotide insertion/deletion at cleavage site. As a result, the edited sequence is most
298 likely to be a mixed population. However, sequencing results have shown that virtually
299 all of edited sequences are end joining product of the two predicted Cas9 cleavage sites.
300 Although additional variations may be discovered when more clones are analyzed, the
301 edited loci often contained only predominant mutant sequence as we have shown
302 previously (33, 39). The reasons for the clonal nature of the appearance of the single
303 population are not fully clear. Whether this is related to the stable expression of the Cas9
304 in these cells or due to other factors require further investigation.

305 The oncogene *v-rel* activates miR-155 expression by binding to NF- κ B site in Bic
306 promoter. We have shown previously that the downregulation of miR-155 in MDV-
307 transformed cell lines could be rescued by expressing *v-rel* in these cells (35). Using the
308 same approach, we have shown here that the downregulation of miR-155 can also be
309 rescued in the context of miR-M4 deleted HP8 by transduction of *v-rel* with RCAS(A)-*v-*
310 *rel*-GFP virus in HP8- Δ miR-M4 clone C48. Interestingly, only 25-fold increase of miR-
311 155 level could be induced in the unedited HP8-Cas9 cells compared to 6,026-fold
312 increase in the miR-M4 deleted clone C48, suggesting that the absence of miR-M4
313 significantly enhances the ability of *v-rel* to induce miR-155 expression in MDV tumor
314 cell lines. As has been demonstrated previously, miR-M4 is highly expressed in MDV
315 tumor cell lines compared to the miR-155, which is actively downregulated, although the
316 precise mechanisms of the differential downregulation have not been identified. Based on

317 the findings from the present study, it appears that the downregulation of miR-155 may
318 be directly linked to high level of miR-M4 expression as the activation of miR-155 by *v-*
319 *rel* is more robust in the miR-M4-deleted cells. However, further studies are required to
320 delineate the associated mechanisms involved in such regulation.

321 The precise editing of the miR-M4 locus to abolish the expression of the mature miR-M4
322 in the MDV-induced T-lymphoma-derived cell line HP8, clearly demonstrated that the
323 proliferative capacity of the transformed cell line is not dependent on continued high
324 level expression of miR-M4. The continued proliferation of cells is unlikely to be due to
325 the inability to express other viral miRNAs such as all other miRNAs in cluster 1 and
326 selected miRNAs from both mid-cluster and LAT-cluster detected by miRNA qRT-PCR
327 (Fig. 4a). MDV-miR-M4 is very important for the oncogenicity of MDV but other
328 miRNAs in the cluster also contribute since the mutant virus expressing miR-M4 alone in
329 cluster 1 remained non-oncogenic (6). Whether or not other miRNA contribute to the
330 maintenance of transformed phenotype remains to be elucidated. The continued
331 proliferation of cells is also not due to the lack of expression of adjacent viral gene such
332 as Meq as we were able to demonstrate the expression of the protein by western blot
333 analysis (Fig. 4b). The significantly increased proliferation capacity of miR-M4 knockout
334 clones suggests that miR-M4 in these context may have proliferation suppressor function.
335 Additional studies on the detailed analysis of the gene expression profiles on these clones
336 will be required to gain further insights into the biology of miR-M4 in these cells.
337 Although it is possible that LCLs may have acquired other mutations that may have made
338 them no longer dependent on miR-M4 for proliferation, the failure on rescuing Meq
339 deleted cell line after repeated attempts indicated this is unlikely the case. Whether or not

340 other genes or miRNAs are involved in maintaining the transformed phenotype of MD
341 tumor cell lines remains to be investigated.

342 **Materials and Methods**

343 **Cell Culture**

344 The MDV-transformed lymphoblastoid cell lines HP8 (40) from a GA strain-induced
345 tumour were grown at 38.5°C in 5% CO₂ in RPMI 1640 medium (Life technologies)
346 containing 10% fetal bovine serum, 10% tryptose phosphate broth, 1% sodium pyruvate
347 solution (Sigma) and 100 units/mL of penicillin and streptomycin (Life technologies).

348 **gRNAs**

349 Two-part guide RNA system containing crRNA:tracrRNA guide complex was used for
350 editing. The sequences of gRNA miR-M4-gN and miR-M4-gC listed in Table 1 were
351 used for synthetic crRNAs production by Integrated DNA Technologies (IDT, USA). The
352 tracrRNA was purchased from IDT. The lyophilized crRNA and tracrRNA pellets were
353 resuspended in Duplex buffer (IDT) at 200 µM concentration and stored in small aliquots
354 at -80°C.

355 **Generation and characterization of HP8-ΔmiR-M4 cell line**

356 NEPA21 Electroporator was used for the transfection of HP8 cells that stably expressed
357 Cas9 (HP8-Cas9) (41). For the deletion of miR-M4, 1x10⁶ of HP8-Cas9 cells were
358 resuspended in 96µL Opti-MEM medium. Two crRNAs miR-M4-gN and miR-M4-gC
359 were mixed with equal molar amounts of tracrRNA to a final duplex concentration of 100
360 µM in 4 µL of duplex buffer and incubated at 95°C for 5 min. After the duplex was
361 allowed to cool to room temperature, it was mixed with cell suspension and
362 electroporated using the conditions of voltage 275V and pulse width 1.5ms of poring

363 pulse. At 48 h post electroporation, 1×10^5 cells were harvested and analysed by PCR. The
364 remaining cells were sorted into 96 wells for single cell isolation. After 7 days
365 incubation, cells were collected and analyzed by PCR. The harvested cell for PCR
366 analysis were lysed in $1 \times$ protein K based DNA isolation buffer (33) at 65°C for 30 min.
367 1 μL of extracted DNA template was used for PCR with primers outside the targeted sites
368 to identify the correct miR-M4 gene knock-out. The primer sequences of miR-M4-F and
369 miR-M4-R used for PCR are listed in Table 1.

370 **RCAS virus infection**

371 Virus stocks were generated from DF-1 cells transfected with RCAS(A)-EGFP and
372 RCAS(A)-*v-rel*-EGFP constructs approximately 5 days after transfection, when 100%
373 cells were EGFP-positive. For *v-rel* transduction in HP8-Cas9 and HP8- $\Delta\text{miR-M4}$, 1 ml
374 ($\sim 10^6$ TCID₅₀) of RCAS(A)-EGFP or RCAS(A)-*v-rel*-EGFP virus stock was used to
375 infect 1×10^6 of HP8- $\Delta\text{miR-M4}$ cells and HP8-Cas9 cells. EGFP expressing RCAS(A)-*v-*
376 *rel*-EGFP and RCAS(A)-EGFP infected HP8- $\Delta\text{miR-M4}$ and HP8-Cas9 cells were sorted
377 into 6 well plates. After 7 days incubation, cells were collected and examined for *v-rel*,
378 EGFP expression by western blot and miR-155 expression by qRT-PCR.

379 **Sorting**

380 For single cell cloning, cells were washed twice with PBS containing 5% FBS and
381 centrifuged at 450g for 5 min at room temperature. The cell pellets were resuspended in
382 cold PBS/5%FBS and sorted into 96 well plate U bottom (Corning) with growth medium
383 by FACS using FACS Aria II (BD bioscience).

384 **qRT-PCR analysis of miRNA expression**

385 The expression level of miRNAs were analyzed using the TaqMan MicroRNA Assay
386 System (Life Technologies) using 10 ng total RNA as a template for reverse transcription.
387 Each reverse transcription reaction was tested by PCR in triplicate and performed twice
388 independently. For relative quantification of miRNA-M4 in HP8-ΔmiR-M4 (Fig. 1d) and
389 miR-155 in *v-rel* transduced cells (Fig. 5b), all values were normalized to the expression
390 of the endogenous let-7a, and levels were calculated as fold-expression change relative to
391 those from HP8-Cas9 cells (miR-155) and CEF (miR-M4). For relative quantification of
392 viral miRNAs and host gga-let-7a in HP8-ΔmiR-M4 clone 48 and controls HP8-Cas9 and
393 CEF (Fig.4a), all values were normalized to the expression of the endogenous GAPDH
394 gene, and levels were calculated as fold-expression change relative to those from CEF.

395 **Dual luciferase reporter assay**

396 Previously constructed reporter construct for the validated miR-M4 and miR-155 target
397 Pu.1 in psiCHECK vector was used to measure the miR-M4 activity in HP8 (28). The
398 reporter constructs contain a 110-bp fragment of the chicken Pu.1 3' untranslated region
399 (UTR) sequence with MRE (Pu.1-3'UTR-wt) or MRE mutant sequence (Pu.1-3'UTR-mu)
400 inserted downstream of Renilla luciferase in the psiCHECK-2 vector (Promega) (28).
401 HP8-ΔmiR-M4 cells and HP8-Cas9 cells (5×10^5) were transfected with 4ug of either
402 Pu.1-3'UTR-wt or Pu.1-3'UTR-mu using NEPA21 electroporator as described. The
403 luciferase expression was assayed 48 h later using the Dual-Glo Luciferase Assay System
404 (Promega) following the manufacturer's instructions. The relative expression of Renilla
405 luciferase was determined with the normalized levels of firefly luciferase. For each
406 sample, values from four replicates representative of at least two independent
407 experiments were used in the analysis.

408 **Western blotting analysis**

409 Approximately 1×10^6 HP8- Δ miR-M4 cells and the control cells were collected and boiled
410 with TruPAGETM LDS sample buffer (Sigma) for 10 min. The samples were separated on
411 a 4-12% TruPAGETM Precast Gel, and the resolved proteins were transferred onto PVDF
412 membranes. Expression of Meq, Pu.1, *v-rel* and GFP was detected using anti-Meq
413 monoclonal antibody (Mab) FD7 (21), rabbit anti-SPIB polyclonal antibody (Aviva
414 Systems Biology), anti-*v-rel* Mab HY87 (35) and GFP Polyclonal Antibody (SICGEN)
415 respectively. α -tubulin (Sigma Aldrich) was used as loading control in all cases. After
416 probing with primary antibodies, the blots were incubated with secondary antibody
417 IRDye@680RD goat anti-mouse IgG (LI-COR) (for Meq, *v-rel* and α -tubulin detection),
418 IRDye@800CW Donkey anti-rabbit IgG (LI-COR) (for Pu.1 detection), IRDye@800CW
419 Donkey anti-goat IgG (LI-COR) (for GFP detection) and visualized using Odyssey Clx
420 (LI-COR). For GFP detection, the PVDF membrane used for *v-rel* detection was stripped
421 and re-probed with GFP antibody following the same procedure.

422 **Analysis of HP8-Cas9- Δ miR-M4 cell growth**

423 The growth of HP8-Cas9- Δ miR-M4 clones along with non-edited HP8-Cas9 was
424 monitored by IncuCyte S3 live cell imaging (Essen Bioscience Ltd, Hertfordshire, UK).
425 Briefly, 8000 cells were seeded in a 96-well plate (Corning) and images were captured
426 every 4h for 132h from four separate regions per well using a 10x objective. By
427 recording the phase object confluence, the growth of HP8-Cas9- Δ miR-M4 clones were
428 compared with parental HP8-Cas9. IncuCyte data was analysed by two-way ANOVA
429 with Tukey's multiple comparisons using GraphPad Prism version 7.01 (GraphPad
430 Software, Inc., San Diego, CA). The results were shown as mean \pm standard error (SE)

431 from four replicates each with 4 separate regions per well representative of three
432 independent experiments. P values of < 0.05 were considered to be significant.

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- 560

561 **Figure legends**

562 **Figure 1. Deletion of the miR-M4 by CRISPR/Cas9 editing in HP8 cells.** (a) The
563 nucleic acid sequences of the truncated/edited PCR products showing the successful
564 deletion of miR-M4 on selected clones. Target sequence is underlined, PAM sequence is
565 in light blue and the cleavage site is indicated by an arrow. (b) The predicated stem loop
566 structure of the pre-miR-M4 with the predicated cleavage site indicated by an arrow. The
567 sequence of the mature miRNA sequences are shown in red. (c) PCR amplification of the

568 edited region using primers NF and CR on the cell lysates of transfected cells at 2 days
569 post transfection and on isolated single cell clones C7-C11. (d) Relative expression of
570 miR-M4, measured by qRT-PCR in RNA extracted from miR-M4 deleted clones C7,
571 C37, C40, and C48 along with the un-edited HP8-Cas9 and CEF. The level of miR-M4 in
572 HP8-Cas9 was set as 1 for calibration.

573 **Figure 2. Proliferation of the HP8-Cas9 and the miR-M4 deleted clones monitored**
574 **in real time using IncuCyte S3 live imaging system.** Cell phase object confluence of
575 each cell population was determined every 4h for 132h from 4 separate regions per well
576 and 4 wells per sample in 96-well plate by IncuCyte and compared with HP8-Cas9
577 control. Growth curves are shown as mean \pm standard error (SE) representative of three
578 independent experiments. Asterisk (*) indicates statistically significant differences
579 between miR-M4 deleted clones and parental HP8-Cas9 cells at different times. *,
580 $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. Asterisks were placed above the
581 time points (single time points) or underneath the growth curves for those time points
582 with the same results during the indicated period of time.

583 **Figure 3. Successful deletion of miR-M4 measured by functional studies.** (a) Firefly
584 and Renilla luciferase activities were measured consecutively with the dual luciferase
585 reporter system (Promega) following transfection of reporter constructs containing the
586 wild-type or the mutant MRE region of the 3' UTR of miR-M4 target gene Pu.1 into the
587 miR-M4 deleted cells and the parental HP8-Cas9. The relative expression of Renilla
588 luciferase was determined with the normalized levels of firefly luciferase. For each
589 sample, values from four replicates representative of at least two independent
590 experiments were used in the analysis. The value from the psiCHECK-2-mutant was set

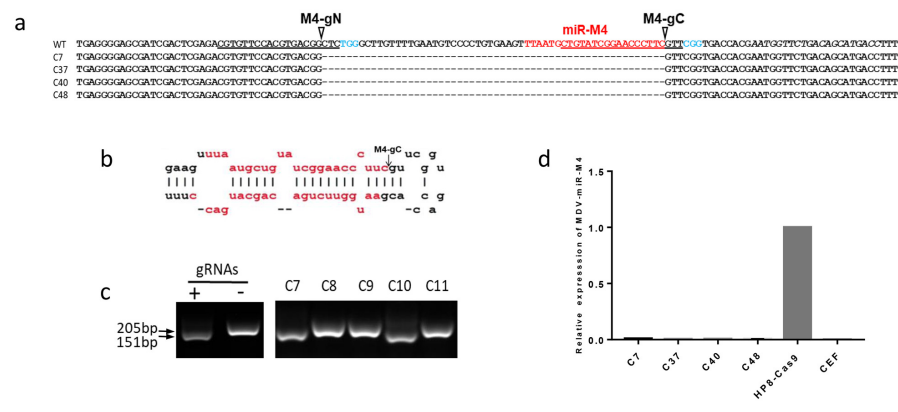
591 as 1. Error bars are derived from four replicates. (b) IP-western blot analysis of Pu.1 in
592 miR-M4 deleted HP8 clone C48 and HP8-Cas9. Matched inputs were assayed for α -
593 tubulin as loading control. Relative signal intensities of the Pu.1 Western blot band were
594 quantified using ImageQuant and normalized against the corresponding signal from the
595 tubulin band. The signal from HP8-Cas9 cells was set as 1.

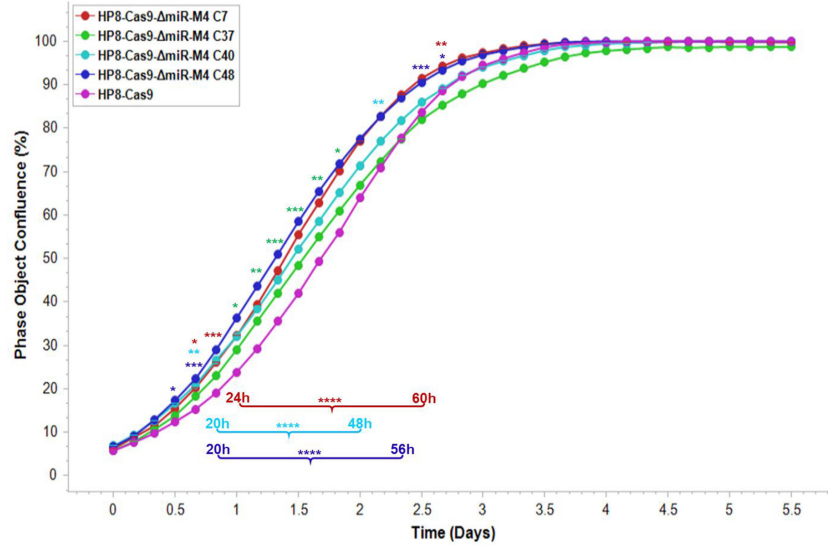
596 **Figure 4. MDV miRNAs and Meq protein expression in miR-M4 deleted cells.** (a)

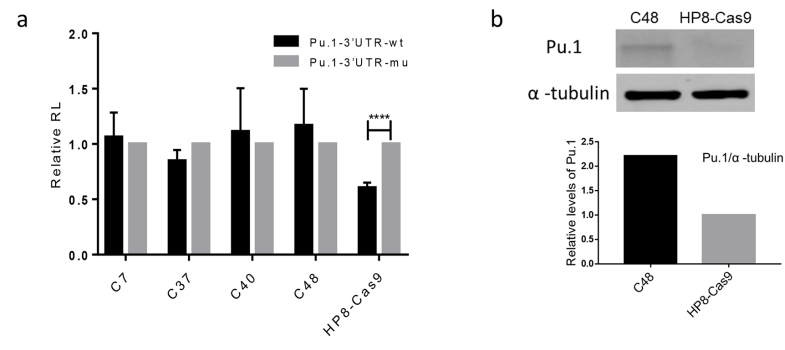
597 Relative expression of each indicated viral miRNAs and host miRNA let-7a is measured
598 by qRT-PCR with RNA extracted from miR-M4 deleted clone C48 along with the un-
599 edited HP8-Cas9 and CEF. All values were normalized to the expression of the
600 endogenous GAPDH gene, and levels were calculated as fold-expression change relative
601 to those from CEF. The level of each miRNA in HP8-Cas9 was set as 1. (b) Detection of
602 Meq expression by western blotting with anti-Meq monoclonal antibody FD7 in HP8-
603 Cas9 and HP8-Cas9- Δ miR-M4 clones. ALV transformed B-cell line HP45 and un-
604 infected CEF were included as negative controls. For the loading control, the same blot
605 was stripped and reprobed with anti- α -tubulin antibody.

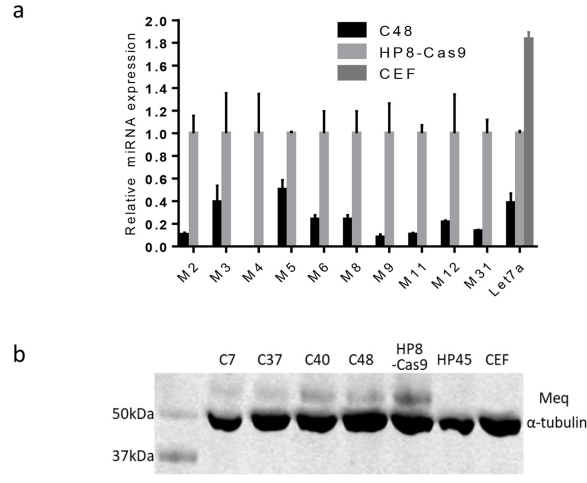
606 **Figure 5. Upregulation of miR-155 in miR-M4 deleted HP8 by v-rel.** (a) Detection of

607 v-rel expression with anti-v-rel monoclonal antibody HY87 and GFP expression with
608 anti-GFP antibody by western blotting in HP8- Δ miR-M4 clone C48 and HP8-Cas9
609 infected with RCAS(A)-GFP or RCAS(A)-v-rel-GFP respectively. For the loading
610 control, the same blot was stripped and reprobed with anti- α -tubulin antibody. (b)
611 Relative level of miR-155 expression was detected by qRT-PCR in HP8- Δ miR-M4 clone
612 C48 and HP8-Cas9 infected with RCAS(A)-GFP or RCAS(A)-v-rel-GFP respectively.









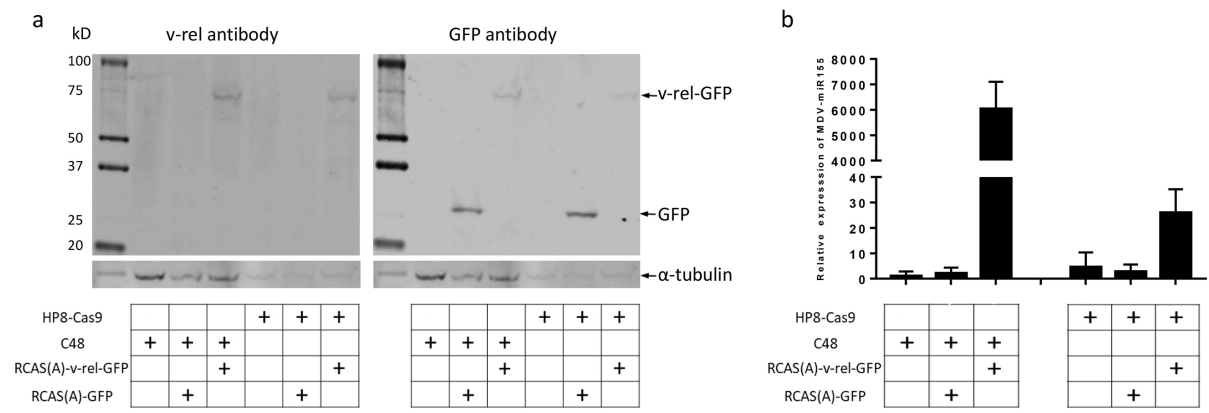


Table 1. List of primer sequences

Primer	Sequence (5'-3')
miR-M4-gN	CGTGTTCACGTGACGGCTC
miR-M4-gC	CTGTATCGGAACCCTTCGTT
miR-M4-F	TGAGGGGAGCGATCGACTC
miR-M4-R	GATTCAATATTACATCACTTCAACGG